Reference: *Biol. Bull.* **178:** 144–159. (April, 1990)

Ontogenetic Change in Digestive Enzyme Activity of Larval and Postlarval White Shrimp *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae)

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Abstract. Whole specimens of developmental stages of *Penaeus setiferus* (Linnaeus, 1767) were homogenized and assayed for activities of digestive enzymes. In all developmental stages, activities were present for trypsin, carboxypeptidase A and B, amylase, and non-specific esterase; none for pepsin or lipase were detected. Activities assayed with substrates for chymotrypsin and aminopeptidase are not apparently due to the presence of these enzymes in the gut. Peak activities for all enzymes occurred during late zoeal or early mysis larval stages; low activities occurred at metamorphosis. During postlarval development, amylase activity increased steadily (by a tenfold increase over five weeks), whereas most other enzyme activities were relatively constant until the fifth week of postlarval development. Although it alters enzyme activity, diet does not appear to be the primary effector of ontogenetic change in digestive enzyme activity. Instead, ontogenetic change in digestive enzyme activity may reflect either a developmentally cued change in enzyme synthesis, or a secondary effect of change in the function and relative size of the midgut during its differentiation.

Introduction

Most previous studies of digestive enzymes in penaeid shrimp (Appendix 1) and other decapod crustaceans have been restricted to adult specimens; interactions between changes in gut morphology, diet, and digestive enzyme activity during early stages of the life cycle are incompletely understood. Penaeid shrimp are ideal crustacean models with which to examine sequential changes in gut structure and function during ontogeny. This is because the ontogeny of these animals is unique among decapod crustaceans: all larval stages are free swimming, rather than embryonated, and transformations to adult morphology and habit are protracted over several weeks, rather than occurring as abrupt transformations in the decapodid stage (PL_1) or in the stages that immediately precede or follow it (Pérez-Pérez and Ros, 1975; Wickins, 1976; Felder *et al.*, 1985; Lovett and Felder, 1989).

After hatching, penaeid larvae pass through five nonfeeding naupliar stages (N_1–N_5), three protozoeal stages (Z_1–Z_3), and three mysis stages (M_1–M_3) before they metamorphose into the decapodid. Within the first two weeks of post-metamorphic life, postlarvae migrate to inshore brackish nursery grounds, adopt a benthic existence, and exhibit a change in feeding habits (Flint, 1956; Pérez-Farfante, 1969; Sastrakusumah, 1970; Jones, 1973; Gleason and Zimmerman, 1984; Gleason, 1986). Experience from aquaculture has shown that this phase of development (PL_1–PL_{14}) represents a "critical period" during which high rates of mortality are encountered (Wickins, 1976; Bages and Sloane, 1981). We hypothesize that this critical period coincides with a change-over in digestive enzyme activity that accommodates the change in habit, so that the shrimp can efficiently digest and assimilate a new diet.

Changes in digestive enzyme activity during development have been studied in relatively few decapod species [*Palaemon serratus:* Van Wormhoudt (1973), Ceccaldi and Trellu (1975), and Van Wormhoudt and Sellos (1980); *Homarus americanus:* Biesiot (1986); and *Penaeus japonicus:* Laubier-Bonichon *et al.* (1977), Gal-

Received 15 May 1989; accepted 22 January 1990.

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gani (1983), and Galgani and Benyamin (1985)]. In most of these cases, assays were conducted for only amylase and general protease activity, and studies were limited to early developmental stages through the decapodid. In the present study, activities for a spectrum of digestive enzymes in *Penaeus setiferus* from early larval stages through the fifth week of postmetamorphic development was measured. These data are evaluated to determine: (1) whether ontogenetic change in digestive enzyme activity is protracted into the postlarval stages (as would be predicted from protracted development of gut morphology); (2) whether ontogenetic change in digestive enzyme activity may reflect ontogenetic change in diet; and (3) whether change in digestive enzyme activity may in part explain occurrence of the "critical period" in development.

Materials and Methods

Specimens examined

Larvae of *Penaeus setiferus* (Linnaeus, 1767) were reared in the laboratory in cylindrical containers (120 l) of natural seawater at 28°C and maintained on a 12:12 light:dark cycle. At stage PL_5 (see Fig. 1 for corresponding carapace length of this and later stages), all shrimp were transferred to plastic tanks (diameter, 1.5 m) filled to 0.1 m depth with continuously aerated seawater. Sand or other natural substrate was not provided. Brood stock was obtained seaward of Freeport, Texas, during July 1986, and spawned within 48 h of capture. Larvae were reared on a diet of algae (*Isochrysis*, *Chaetocerus*, and *Tetraselmis*) and 24-h *Artemia* nauplii (Aquacop, 1983; McVey and Fox, 1983). Beginning with PL_5, the diet consisted entirely of *Artemia* nauplii (Great Salt Lake Brand®, Sanders Brine Shrimp Co., Ogden, Utah).

Larval stage was identified in accord with descriptions by McVey and Fox (1983). Postlarval stages were identified by postmetamorphic age, as is the practice in culture of penaeid shrimp. Specimens were isolated in a brass sieve of appropriate mesh size, blotted dry, weighed, and placed in −70°C ultracold freezer until assays were conducted. Fresh samples were also assayed to monitor the effect of freezing on enzyme activity. Two separate spawns were examined; results from each spawn were not combined. To compare enzyme activities of early postlarval stages with those of juveniles, one brood was reared to PL_{140}. Individual juvenile shrimp were assayed as single samples. Samples of *Artemia* (but not algae) were also assayed.

Because diel rhythmicity in enzyme activity has been reported for adults of *Penaeus* (Van Wormhoudt *et al.*, 1972; Van Wormhoudt, 1973; Ceccaldi, 1981; Cuzon *et al.*, 1982; Maugle *et al.*, 1982b), samples for assays of all postlarval specimens, beginning with stage PL_4, were collected in mid-morning when peak enzyme activities reportedly occur. However, because duration of each larval stage is relatively short, samples of larvae and PL_1 were collected when 80% of the population had attained the desired stage, irrespective of time of day. Temperature, photoperiod, salinity, and time of day that food was presented were held constant to reduce variability in enzyme activity. Because ecdysis is not entirely synchronous and because it would not have been feasible to sort by molt stage the large number of specimens required for each sample, it was not possible to control stage in molt cycle.

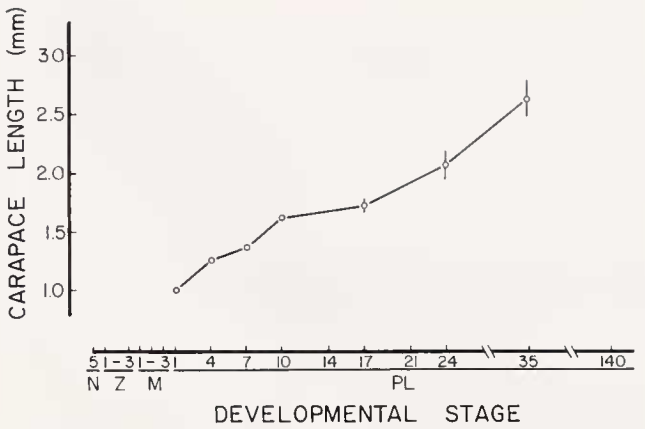

Figure 1. Average carapace length (excluding rostrum) for postlarval stages of *Penaeus setiferus* used in enzyme assays. Error bars indicate 95% confidence interval about mean carapace length of each developmental stage (n = 30). Average carapace length of PL_{140} was 3.42 ± 0.18 mm (n = 15). N, nauplis stage 5; Z, protozoeal stages 1–3; M, mysis stages 1–3; PL, age of postlarvae in days.

To determine whether activity assayed in whole-animal homogenates came primarily from gut tissues or instead from enzymes in other tissues of the shrimp, the entire gut was dissected from specimens of juvenile shrimp. The remaining carcass was rinsed and blotted dry. The gut and carcass were frozen at −70°C and assayed independently.

Assays

Whole-animal homogenates were used in all assays. Samples were homogenized in 9 volumes of 0.05 *M* Tris (hydroxymethyl) aminomethane hydrochloride buffer, pH 7.8, with 0.011 *M* $CaCl_2$ in a Wheaton ground-glass tissue grinder. Homogenates were centrifuged at 4800 × *g* for 60 min at 4°C in accord with Lee's (1984) method. The supernatant, exclusive of the lipid layer, was collected and stored at 4°C until assayed. All enzyme assays were conducted within 20 h of homogenization.

Duplicate assays were conducted for each sample, and the mean value was used in calculations. Total soluble protein was measured with the Peterson (1977) modification of the Lowry *et al.* (1951) method, but with human serum albumin as a standard (bovine serum albumin is subject to surface denaturation in dilute solutions).

Tryptic enzyme activity was assayed using two methods: 1.00 m*M* α-p-toluenesulphonyl-L-arginine methyl ester hydrochloride (TAME) at pH 8.1, 25°C by the method of Hummel (1959) as adapted by Rick (1974b), and 0.417 m*M* N-α-benzoyl-DL-arginine ρ-nitroanilide hydrochloride (BAPNA) at pH 8.2, 25°C, by the method of Erlanger *et al.* (1961). Because some inhibition of BAPNA hydrolysis was observed in early developmental stages, substrate concentration was reduced below that suggested by Erlanger *et al.*

Carboxypeptidase A was assayed with 0.35 m*M* hippuryl-L-phenylalanine at pH 7.6, 37°C, by the method of Folk and Schirmer (1963) as modified by Appel (1974). Carboxypeptidase B was assayed with 0.352 m*M* hippuryl-L-arginine at pH 7.6, 37°C, by the method of Folk *et al.* (1960) as modified by Appel (1974).

Chymotryptic-like esterase activity was assayed with 0.342 m*M* N-benzoyl-L-tyrosine ethyl ester (BTEE) at 25°C, pH 7.8, by the method of Rick (1974a). Assays of either fresh or frozen specimens with glutaryl-L-phenylalanine-ρ-nitroanilide (GPANA) by the method of Erlanger *et al.* (1964) did not yield activity that differed significantly from controls.

Arylamidase ("aminopeptidase") activity was assayed with 0.7 m*M* L-leucine-ρ-nitroanilide (LPNA) at pH 7.8, 25°C, by the method of Binkley and Torres (1960) as modified by Appel (1974). To determine whether this activity was membrane-associated in *P. setiferus*, both homogenate with buffer and homogenate with 1% Triton-X 100 were centrifuged at 100,000 × *g* for 1 h. Total activity was not significantly different in the two resulting supernatants or in the original homogenate. No activity was found in resuspended pellets. Assays with L-leucinamide hydrochloride, by the method of Binkley and Torres (1960), or with L-leucyl-β-naphthylamide hydrochloride, by the method of Burstone and Folk (1956), did not yield activities significantly different from controls.

Non-specific esterase and lipase activities were measured with three separate substrates at pH 7.4 by the method of Nachlas and Seligman (1949): 0.4603 m*M* β-naphthyl acetate (C_2) at 25°C for 20 min, 0.2625 m*M* β-naphthyl laurate (C_{12}) at 37°C for 60 min, and 0.2088 m*M* β-naphthyl stearate (C_{18}) at 37°C for 180 min. Cholinesterase activity was inhibited by addition of 10^{-5} *M* eserine to substrate solution. Activity was determined from a standard curve of β-naphthol absorbance. Although a titrimetric technique with tributyrin may be a preferred method for assay of lipase activity (Desnuelle, 1972), limited sample volume precluded application of such a method in the present study. Assay of lipase activity with the copper method of Schmidt *et al.* (1974) did not yield activity significantly different from controls.

Amylase activity was assayed with two separate substrates at pH 6.9 with 0.01 *M* NaCl, 25°C: 1.0% purified potato starch solution (4.634 mg/ml in reaction mixture) and 0.66% purified oyster glycogen (3.059 mg/ml) (obtained from Sigma Chemical Co., St. Louis, Missouri). Maltose released by hydrolysis of substrate was measured by reaction with dinitrosalicylic acid reagent by the method of Bernfeld (1955) as modified by Rick and Stegbauer (1974). Activity was determined from a standard curve of D (+) maltose absorbance. To exclude activity due to disaccharidases in the homogenate, a separate assay was conducted in which maltose was the substrate; this disaccharidase activity (which was very low) was subtracted from total activity measured in amylase assays.

Peptic activity was measured with N-acetyl-L-phenylalanine-L-3,5-di-iodotyrosine by the method of Rick and Fritsch (1974). Activity did not differ significantly from controls.

Amylase/protease ratio

The ratio of amylase activity to protease activity (A/P ratio), frequently used to characterize digestive capability (for example, Laubier-Bonichon *et al.*, 1977; Lee *et al.*, 1980; Van Wormhoudt *et al.*, 1980), was estimated from the ratio of amylase activity (starch as substrate) to trypsin activity (BAPNA as substrate). Because trypsin accounts for 40–50% of the proteolytic activity in adult penaeids (Galgani, 1983; Galgani *et al.*, 1984), we feel justified in substituting activity of this enzyme for total protease activity in the calculation.

Estimation of activity in hepatopancreas

While digestive enzyme activity is often determined as activity per mg soluble protein in the hepatopancreas, isolation of the hepatopancreas from larvae and small postlarvae is impractical. Therefore, the ratio of hepatopancreas volume to total body volume was used to estimate enzyme activity per gram wet weight of hepatopancreas. The ratio of hepatopancreas density to density of non-gut tissue, and the ratio of lipid content to protein content of tissues, were assumed to be similar in all developmental stages. For each developmental stage, the hepatopancreas and total body volume were estimated from reconstructed serial sections (8 μm) of formalin-fixed, paraffin-embedded specimens. The area of each section was measured, and the volume was calculated by

Table I

Effect of freezing on digestive enzyme activities in homogenates of Penaeus setiferus

Enzyme (Substrate)	Ratio of frozen to fresh activity (range)
Trypsin (BAPNA)	1.03 (0.91–1.18)
Carboxypeptidase A	0.63* (0.53–0.68)
Carboxypeptidase B	1.47* (1.10–1.54)
Esterase (β-naphthol acetate)	2.27* (1.67–3.57)
Esterase (β-naphthol laurate)	2.04* (1.67–2.17)
Amylase (starch)	0.81 (0.69–1.02)

Effect expressed as ratio of specific activity of enzyme in previously frozen samples to specific activity in fresh samples. Values reported are averages of ratio calculated for all developmental stages compared. Assays for trypsin (TAME), non-specific esterase (β-naphthol stearate), and amylase (glycogen) were not conducted for fresh specimens.

* = Indicates that difference in activity between frozen and fresh samples was significantly different ($P < 0.05$) within respective developmental stages.

summing frusta. The average ratio, of soluble protein content of hepatopancreas to soluble protein content of the carcass in juveniles (5.43), was used to estimate enzyme activity per mg protein in the hepatopancreas. Protein content of tissues was assumed to be constant across all developmental stages, and all measured activity was assumed to have come from the hepatopancreas.

Results

None of the enzymes were inactivated completely by freezing, although carboxypeptidase A activity was diminished significantly ($P < 0.05$) (Table I). Carboxypeptidase B and non-specific esterase activities were significantly higher ($P < 0.05$) in frozen than in fresh specimens. Ratio of the activity in frozen specimens to that in fresh specimens was similar for each enzyme for all larval and postlarval stages. Furthermore, enzyme activity in samples frozen for 48 hours was not significantly different ($P < 0.05$) from activity in samples frozen for 6 months.

Most chymotryptic-like esterase activity, and a substantial proportion of arylamidase activity, came from non-gut tissues (Table II). There also was a small amount of activity for trypsin (with BAPNA as substrate) and non-specific esterase (with β-naphthol acetate as substrate) in non-gut tissues. For all other enzyme assays (including the trypsin assay with TAME as substrate), activity measured in whole-animal homogenates can be attributed largely to gut tissue. In addition, enzyme activities in *Artemia* nauplii (Table III) were always sufficiently low to exclude the possibility that a substantial proportion of enzyme activity measured in *P. setiferus* originated from ingested *Artemia*.

Because total protein per gram wet weight influences specific enzyme activity, it was important to determine whether ontogenetic change in soluble protein content of whole-animal homogenates occurred. In fact, relative protein content remained essentially constant through all developmental stages, except in the first two larval stages examined (Fig. 2). These two larval stages are highly setose and dense setae may have precluded removal of adherent water when samples were blotted dry.

Three general patterns of ontogenetic change in enzyme activity were observed: (1) proteases (Fig. 3)—activities are low in N_5, increase to a peak at Z_3, decrease to a low around PL_1, remain somewhat low until about PL_{17}, and thereafter increase slightly through the remainder of postlarval development; (2) esterases (Fig. 4)—activities are low in N_5, increase to a peak at Z_1, decrease to a low at M_3, and thereafter increase slightly or stabilize during the remainder of postlarval development; and (3) amylase (Fig. 5)—activity is relatively low in N_5, increases to a peak at M_2, decreases to a low at PL_1–PL_4, and thereafter increases markedly through the remainder of postlarval development. The pattern for esterases is similar to that of proteases, except that changes in esterase activity precede changes in protease activity by one stage. The A/P ratio (Fig. 6) is high in N_5 and M_2, but low at Z_3. An increase in the A/P ratio begins at PL_7 and continues until PL_{24}.

Significant ontogenetic decrease in the ratio of hepatopancreas volume to volume of the whole body occurs during larval and early postlarval development (Fig. 7). By about PL_7, this ratio reaches a low value and remains unchanged during the remainder of postlarval development; postlarval body size increases at a relatively constant rate throughout postlarval development (Fig. 1).

In the three patterns described above, enzyme activity is expressed as activity per mg soluble protein in the whole shrimp. When activity of enzymes in *P. setiferus* is corrected to reflect developmental change in the ratio of hepatopancreas volume to total body volume (see Materials and Methods) and is expressed as a function of soluble protein in the hepatopancreas (Fig. 8), patterns for ontogenetic change in enzyme activity differ considerably from those described above. Activities of proteases are low at N_5, and increase slightly to peaks in the middle stages of larval development. However, activities of these enzymes remain relatively constant during postlarval development until PL_{21}–PL_{28} and then increase at PL_{35}. Amylase activity is essentially constant (and low) during larval development, but after PL_4, activity increases steadily through development. Only in esterolytic enzymes is there a peak in activity at N_5. Activity decreases dramatically during the mysis stages, and increases only slightly during postlarval development.

Table II

Enzyme activity assayed separately in gut and non-gut tissues of Penaeus setiferus *juveniles*

Enzyme (substrate)	Activity (IU/g wet weight)			Activity (IU/mg protein)		
	Gut	Carcass	Ratio	Gut	Carcass	Ratio
Trypsin (BAPNA)	0.207 (±0.094)	0.023 (±0.038)	9.0	0.0527 (±0.0235)	0.0010 (±0.0017)	52.3
Trypsin (TAME)	29.7 (±12.2)	1.17 (±1.43)	25.4	7.53 (±2.31)	0.05 (±0.07)	150.6
Carboxypeptidase A	3.08 (±0.67)	0.13 (±0.055)	23.7	0.787 (±0.258)	0.006 (±0.003)	131.2
Carboxypeptidase B	5.90 (±1.76)	0.27 (±0.25)	21.8	1.504 (±0.498)	0.013 (±0.011)	115.7
Chymotryptic-like esterase	0.27 (±0.10)	3.95 (±4.15)	0.1	0.067 (±0.022)	0.184 (±0.216)	0.4
Arylamidase	0.204 (±0.205)	0.136 (±0.204)	1.5	0.0517 (±0.0488)	0.0064 (±0.0093)	8.0
Esterase (β-naphthol acetate)	0.188 (±0.052)	0.018 (±0.016)	10.4	0.0478 (±0.0072)	0.0007 (±0.0006)	68.3
Amylase (starch)	21.5 (±3.7)	0.5 (±0.6)	43.0	5.47 (±1.01)	0.02 (±0.03)	273.5

Ratio of activity in gut tissues to activity in remaining carcass indicated. Activity expressed both as International Units of activity per gram wet weight and International Units of activity per mg protein. Mean activity (±95% confidence limit) is indicated (n = 3).

Discussion

The ontogenetic decrease in specific activities of digestive enzymes at metamorphosis coincides with degeneration of the gut (from M_1–PL_4) in *Penaeus setiferus*. The subsequent increase in enzyme activities during postlarval development coincides with differentiation of the gut into the adult form. This increase represents both an increase in enzyme activities in hepatopancreatic tissues, and an allometric increase in the relative size of the hepatopancreas. Observed changes in enzyme activities during postlarval development are not the result of change in diet because diet was held constant during this period. Thus, the ontogenetic change in activities represents some other change associated with development.

Table III

Specific activities for digestive enzymes for 24-h Artemia *nauplii, obtained from whole-animal homogenates*

Enzyme (Substrate)	Specific activity (IU/mg protein)
Trypsin (BAPNA)	0.0055 ± 0.0012
Trypsin (TAME)	0.27 ± 0.08
Carboxypeptidase A	0.034 ± 0.013
Carboxypeptidase B	0.20 ± 0.02
Non-specific esterase (β-naphthol acetate)	0.023 ± 0.005
Non-specific esterase (β-naphthol laurate)	0.0016 ± 0.0002
Non-specific esterase (β-naphthol stearate)	0.00014 ± 0.00002
Amylase (starch)	0.100 ± 0.004
Amylase (glycogen)	0.038 ± 0.018

Activities expressed as International Units of activity per mg protein. Mean activity ± 95% confidence limit is indicated for three replicates.

Ontogenetic change in digestive enzyme activity

The ontogenetic patterns of enzyme activity found in *Penaeus setiferus* are similar to those described for other decapod species (Van Wormhoudt, 1973; Laubier-Bonichon *et al.*, 1977; Van Wormhoudt and Sellos, 1980; Galgani and Benyamin, 1985; Biesiot, 1986). In *P. setiferus, P. japonicus, Palaemon serratus,* and *Homarus americanus,* specific activities of both amylase and protease are low in those developmental stages preceding the first

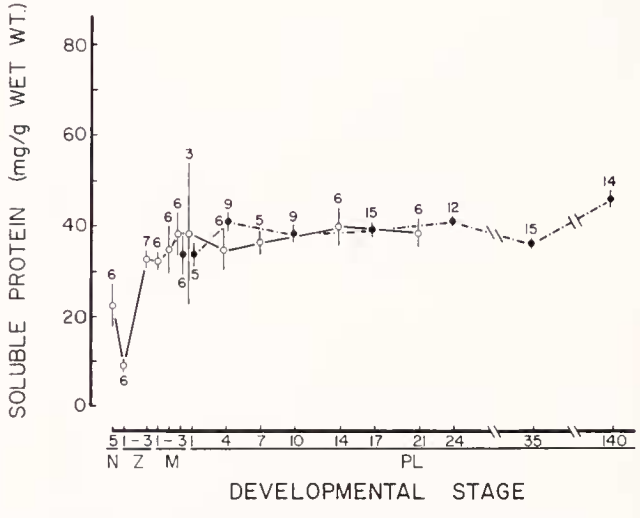

Figure 2. Soluble protein content in developmental stages of *Penaeus setiferus*, obtained from whole-animal homogenates. Solid *versus* broken lines indicate separate spawnings. Error bars indicate 95% confidence interval about mean value for each developmental stage. Sample size for each mean is indicated by numbers above or below bars. N, nauplis stage 5; Z, protozoeal stages 1–3; M, mysis stages 1–3; PL, age of postlarvae in days.

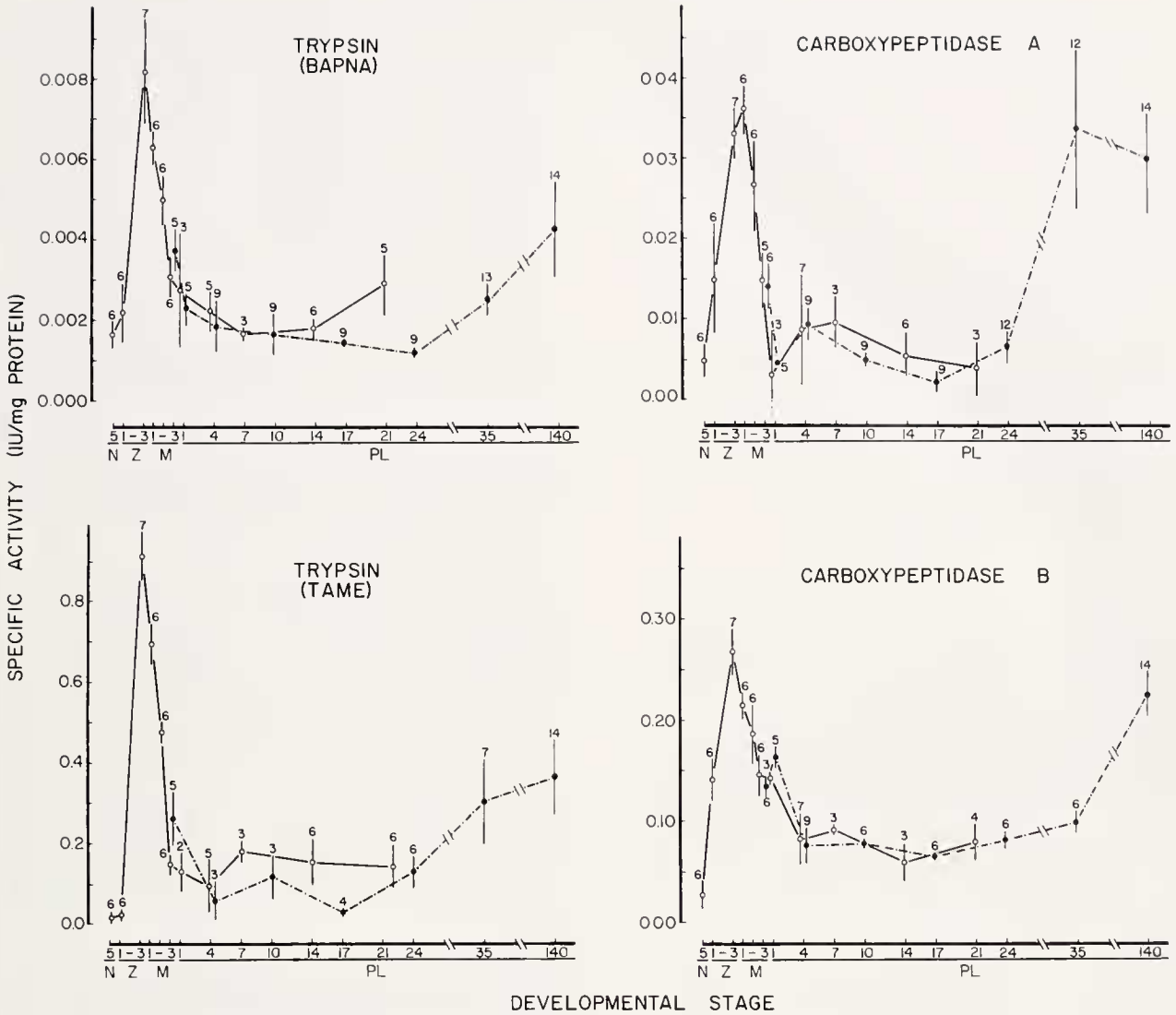

Figure 3. Specific activity of trypsin (with substrate indicated), carboxypeptidase A, and carboxypeptidase B for developmental stages of *Penaeus setiferus*, obtained from whole-animal homogenates. Activity expressed as International Units of activity per mg protein in entire animal. Solid *versus* broken lines indicate separate spawnings. Error bars indicate 95% confidence interval about mean activity for each developmental stage. Sample size for each mean is indicated by numbers above or below bars. N, nauplius stage 5; Z, protozoeal stages 1–3; M, mysis stages 1–3; PL, age of postlarvae in days.

feeding stage. Enzyme activities increase during early larval development in all four species. In *P. setiferus* and *P. japonicus*, amylase activity decreases to a low level by metamorphosis, but activity remains relatively constant in *P. serratus* and *H. americanus*. In all species, amylase activity increases during postlarval development. Protease activity in all four species decreases at metamorphosis. During early postlarval development, protease activity remains low in *Penaeus* spp., but increases in *P. serratus* and *H. americanus*. There is a peak in A/P ratio for *P. japonicus* at Z_3, but in *P. setiferus* the peak occurs at M_2; in both species the ratio declines to a low level at metamorphosis.

Ontogeny of gut. Increase in digestive enzyme activity has been correlated with differentiation of the gut in larvae of both teleosts (Buddington and Doroshov, 1986) and echinoderms (Vacquier *et al.*, 1971). In *P. setiferus*, the decrease in most enzyme activities immediately after metamorphosis coincides with degeneration of the anterior midgut caeca into the vestigial anterior midgut diverticulum (Lovett and Felder, 1989). Laubier-Bonichon *et al.* (1977) examined whole-animal concentra-

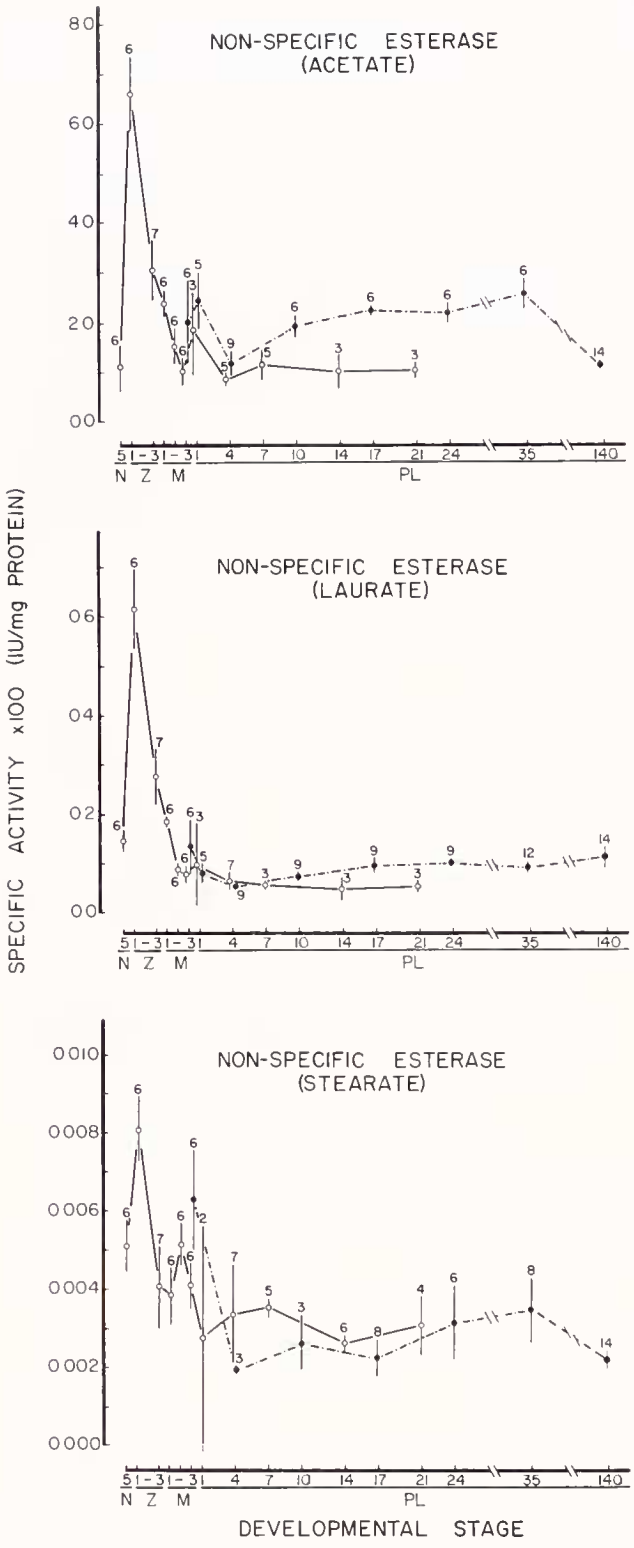

Figure 4. Specific activity of non-specific esterase (with fatty acid chain of β-naphthol substrate indicated) for developmental stages of *Penaeus setiferus*, obtained from whole-animal homogenates. Activity expressed as International Units of activity per mg protein in entire animal. Solid *versus* broken lines indicate separate spawnings. Error bars indicate 95% confidence interval about mean activity for each developmental stage. Sample size for each mean is indicated by numbers above or below bars. N, nauplius stage 5; Z, protozoeal stages 1–3; M, mysis stages 1–3; PL, age of postlarvae in days.

tions of RNA and DNA in *P. japonicus* and concluded that the rates of cell multiplication, cell hypertrophy, and cellular metabolic rate were at peak levels during larval development, but then dropped to low levels during the first week of postlarval life. Thus, reduced metabolic activity during the critical period may coincide with low digestive enzyme activity observed, and may reflect some accommodation to limited nutrient uptake during this transformational period of morphogenesis in the gut.

The increase in enzyme activities following the critical period in *P. setiferus* coincides with the ramification of lobes of the hepatopancreas into small-diameter tubules (Lovett and Felder, 1989). In *Palaemon serratus*, the increase in enzyme activity also coincides with an increase in the number of caeca in the hepatopancreas (Van Wormhoudt, 1973; Richard, 1974, thesis cited in Van Wormhoudt and Sellos, 1980), but unlike the situation in *P. setiferus*, the increase in number of caeca in *P. serratus* occurs during larval development. In *P. setiferus*, substantial increases in enzyme activities (particularly for trypsin, carboxypeptidase A, and amylase) occur during the fourth and fifth week of postlarval development and coincide with completion of differentiation by the hepatopancreas. Moreover, by this stage in development, the foregut has nearly attained the adult morphology and function, the posterior diverticulum has differentiated, and retention time of food in the gut has increased dramatically over that of early postlarval stages (Lovett and Felder, 1989, 1990). Thus, protraction in development of gut morphology of *P. setiferus* is reflected in the protraction of ontogenetic change in digestive enzyme activity.

Ontogeny of feeding habits. Ontogenetic change in enzyme activity can also be correlated with diet and feeding habit. In stage Z_1, larvae of *P. setiferus* begin to feed on algae; esterase activity is maximal. We provided *Artemia* nauplii beginning at stage M_1 (although larvae of *P. setiferus* will feed on *Artemia* beginning at stage Z_3); activities of trypsin and carboxypeptidase A and B in larvae are maximal at Z_3–M_1. Larvae shift from being primarily filter feeders to being primarily raptorial feeders at M_3, and filtering efficiency continues to decline during PL_1 (Emmerson, 1980, 1984); enzyme activities decline during M_3 and become very low at PL_1. The diet in wild populations is reported to change from predominately algae in early postlarval stages to include a more substantial portion of animal matter in later (PL_{28}–PL_{35}) postlarval stages (Flint, 1956; Fujinaga, 1969; Sastrakusu-

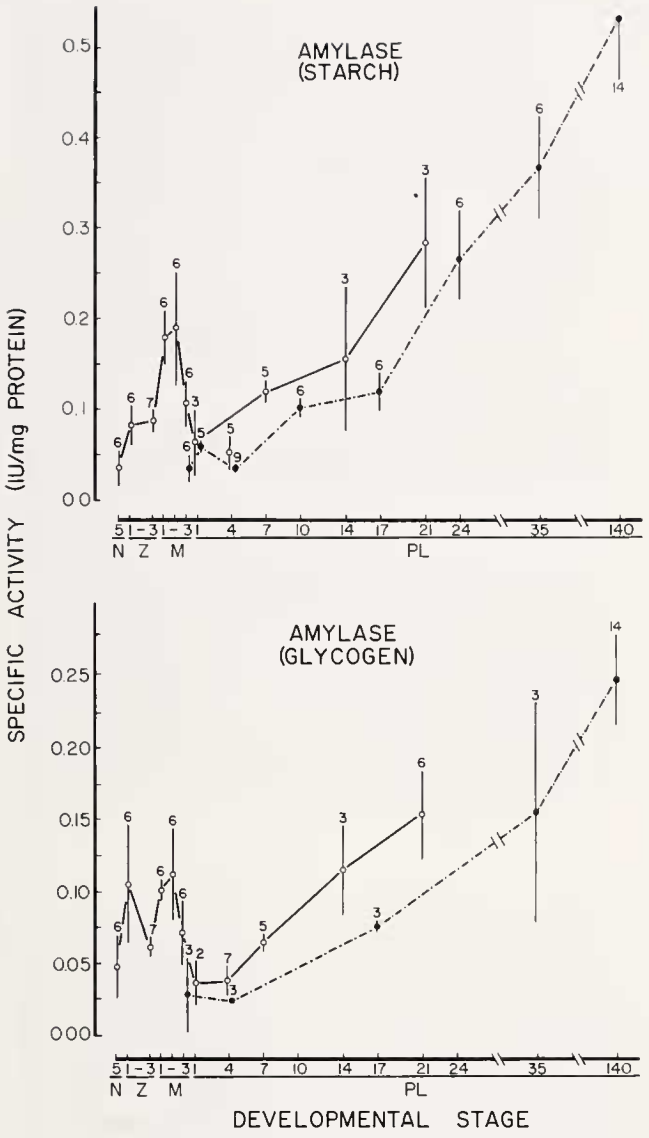

Figure 5. Specific activity of amylase (with substrate indicated) for developmental stages of *Penaeus setiferus*, obtained from whole-animal homogenates. Activity expressed as International Units of activity per mg protein in entire animal. Solid *versus* broken lines indicate separate spawnings. Error bars indicate 95% confidence interval about mean activity for each developmental stage. Sample size for each mean is indicated by numbers above or below bars. N, nauplius stage 5; Z, protozoeal stages 1–3; M, mysis stages 1–3; PL, age of postlarvae in days.

mah, 1970; Jones, 1973; Chong and Sasekumar, 1981; Nelson, 1981; Gleason and Zimmerman, 1984; but see also Kitting *et al.*, 1984; Gleason, 1986). In PL_{28}–PL_{35}, enzyme activity increases substantially.

Despite the correlation of ontogenetic change in enzyme activity with change in feeding habits, ontogenetic change in activity may be developmentally cued and may reflect temporal genetic regulation of enzyme synthesis, rather than a change in diet. For example, ontogenetic change of digestive enzyme activity in the first feeding stages of *Homarus* larvae occurs even in the absence of access to exogenous food substrates (Biesiot, 1986). In *Artemia*, ontogenetic change in enzyme synthesis is likely under genetic control, which then is modulated by diet and nutritional requirements (Samain *et al.*, 1980). Moreover, no consistent correlation of A/P ratio with composition of diet has been found within a single crustacean species (Hoyle, 1973; Boucher *et al.*, 1976; Samain *et al.*, 1980; Maugle *et al.*, 1982b; Båmstedt, 1984; Harris *et al.*, 1986). Because both amylase activity and the A/P ratio in *P. setiferus* increase during postlarval development, it might be inferred that postlarval shrimp become more herbivorous. However, beginning with PL_5, the diet consisted entirely of *Artemia*. Thus, change in enzyme activity in *P. setiferus* occurs without a change in diet.

Dietary implications

Even though we did not vary diet in the present study, we can infer that diet is not the only factor influencing enzyme activity. Attempts have been made to correlate digestive enzyme activity with diet and to use ontogenetic change in enzyme activity as an index of trophic state to estimate the phase in development where diet formulations for cultured shrimp need to be changed

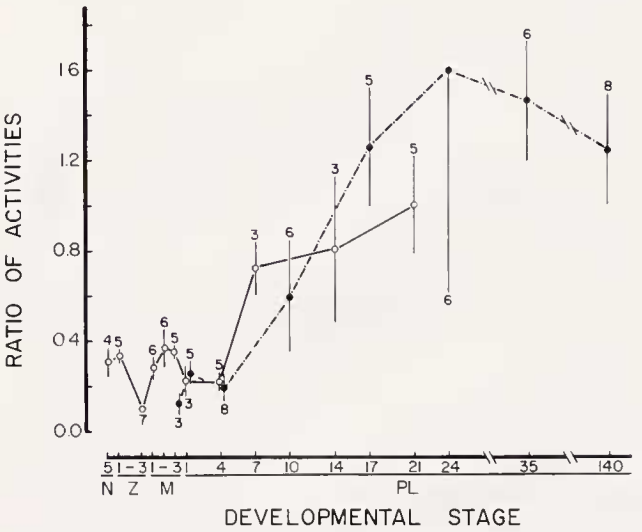

Figure 6. Ratio of amylase activity (starch used as substrate) to trypsin activity (BAPNA used as substrate), for developmental stages of *Penaeus setiferus*. Solid *versus* broken lines indicate separate spawnings. Error bars indicate 95% confidence interval about mean activity for each developmental stage. Sample size for each mean is indicated by numbers above or below bars. N, nauplius stage 5; Z, protozoeal stages 1–3; M, mysis stages 1–3; PL, age of postlarvae in days.

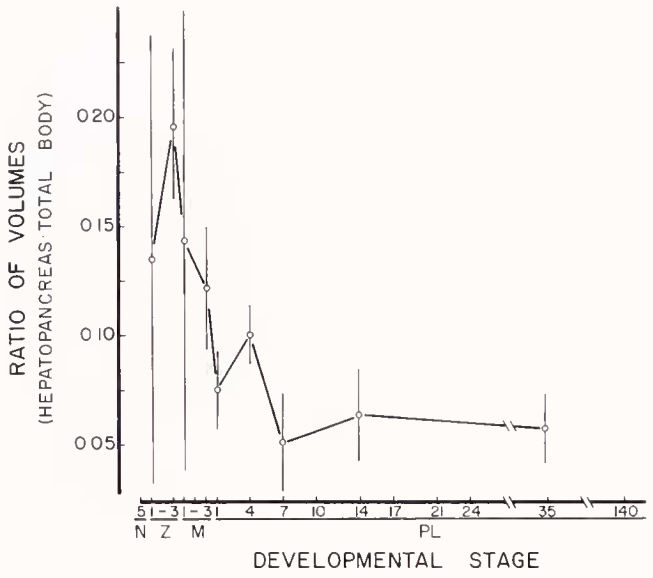

Figure 7. Ratio of volume of hepatopancreas (in early stages, volume of anterior midgut caeca plus lateral midgut caeca) to total body volume for developmental stages of *Penaeus setiferus*. Areas were measured on 8 μm serial sections of formalin-fixed, paraffin-embedded specimens. Volumes were calculated by summing frusta. Error bars indicate 95% confidence interval about mean ratio of volumes are indicated for each developmental stage (n = 3). N, nauplius stage 5; Z, protozoeal stages 1–3; M, mysis stages 1–3; PL, age of postlarvae in days.

(Hoyle, 1973; Van Wormhoudt, 1973; Laubier-Bonichon *et al.*, 1977; Cuzon *et al.*, 1980; Lee *et al.*, 1980, 1984; Van Wormhoudt *et al.*, 1980; Maugle *et al.*, 1982b; Galgani, 1983; Galgani *et al.*, 1984; Lee and Lawrence, 1985). Despite postlarval diet being held constant in the present study, a significant ontogenetic change occurred in digestive enzyme activity and in the A/P ratio. Therefore, we question the validity of using the A/P ratio to predict the degree to which an organism is carnivorous or herbivorous. In those larval stages of *Penaeus* with maximal amylase activity and A/P ratio, the diet is indeed composed of phytoplankton. However, the significance of high amylase activity here is not clear; very few groups of marine phytoplankton use starch as a storage product, and those storage products used most widely by marine phytoplankton are not hydrolyzed by amylase.

In contrast to the usual explanation for diet effects on enzyme activity, Harris *et al.* (1986) and Hofer (1982) propose that secretion of large amounts of enzyme may maximize the use of a scarce component in the diet. Such elevated enzyme activity could maximize hydrolysis and the resulting extraction of a dietary substrate that was present in small amounts. Thus, the substantial increase in amylase activity observed in *P. setiferus* during postlarval development may be a response to low levels of carbohydrate in the postlarval diet. A similar response to elimination of starch from the diet was observed by Hernandorena (1982) in *Artemia*. However, such a response is contrary to an assumption that is widely held among aquaculturists: *i.e.*, that enzyme activity is high for those substrates most common in the diet.

An additional problem associated with the use of enzyme activity to evaluate diet is that the stage of molt cycle, nutritional status of shrimp, season, sexual condition, and ontogenetic stage have been shown to affect size, histological condition, water content, and protein content of the hepatopancreas, (Cuzon *et al.*, 1980; Rosemark *et al.*, 1980; Van Wormhoudt *et al.*, 1980; Van Wormhoudt and Sellos, 1980; Storch *et al.*, 1982; Barclay *et al.*, 1983; Pascual *et al.*, 1983; Storch and Anger, 1983; Lee, 1984; Lee *et al.*, 1984; Storch *et al.*, 1984; Vogt *et al.*, 1985). Thus, the units selected to express enzyme activity (either activity per mg protein in hepatopancreas, activity per g wet weight of hepatopancreas, activity per mg protein in whole shrimp, or activity per g wet weight of shrimp) can affect whether significant change in digestive enzyme activity is reported (Cuzon *et al.*, 1980; Van Wormhoudt *et al.*, 1980; Barclay *et al.*, 1983; Lee, 1984; Lee *et al.*, 1984; present study). Little is known about the implications of using any one of these units to describe enzyme activity in shrimp.

Enzymes present in gut

Lack of specificity in assay substrates precludes conclusive identification of enzymes responsible for hydrolysis of substrates. For example, the substrates TAME and BAPNA are specific for trypsin only in the sense that they are not hydrolyzed by chymotrypsin (Hummel, 1959; Rick, 1974b). They can be hydrolyzed by both non-specific esterases and crustacean collagenase (Hess and Pearse, 1958; Pearse, 1972; Grant and Eisen, 1980; Grant *et al.*, 1983). The chymotrypsin-specific substrate BTEE also is subject to hydrolysis by both non-specific esterase and Type I crustacean collagenase, whereas GPANA is relatively resistant to hydrolysis by either of these enzymes (Eisen *et al.*, 1973; DeVillez, 1975). Chymoptryptic-like activity is not considered further in the present study because: (1) no activity was measured with GPANA, (2) most activity measured with BTEE occurred in non-gut tissues, and (3) conclusive evidence that crustaceans secrete chymotrypsin in quantities significant for digestion is lacking (DeVillez, 1975; Vonk and Western, 1984; Appendix 1). Both arylamidase and aminopeptidase are not considered further because: (1) no activity was found with either of the aminopeptidase substrates L-leucinamide or L-leucyl-β-naphthylamide as substrates, (2) activity measured with the substrate

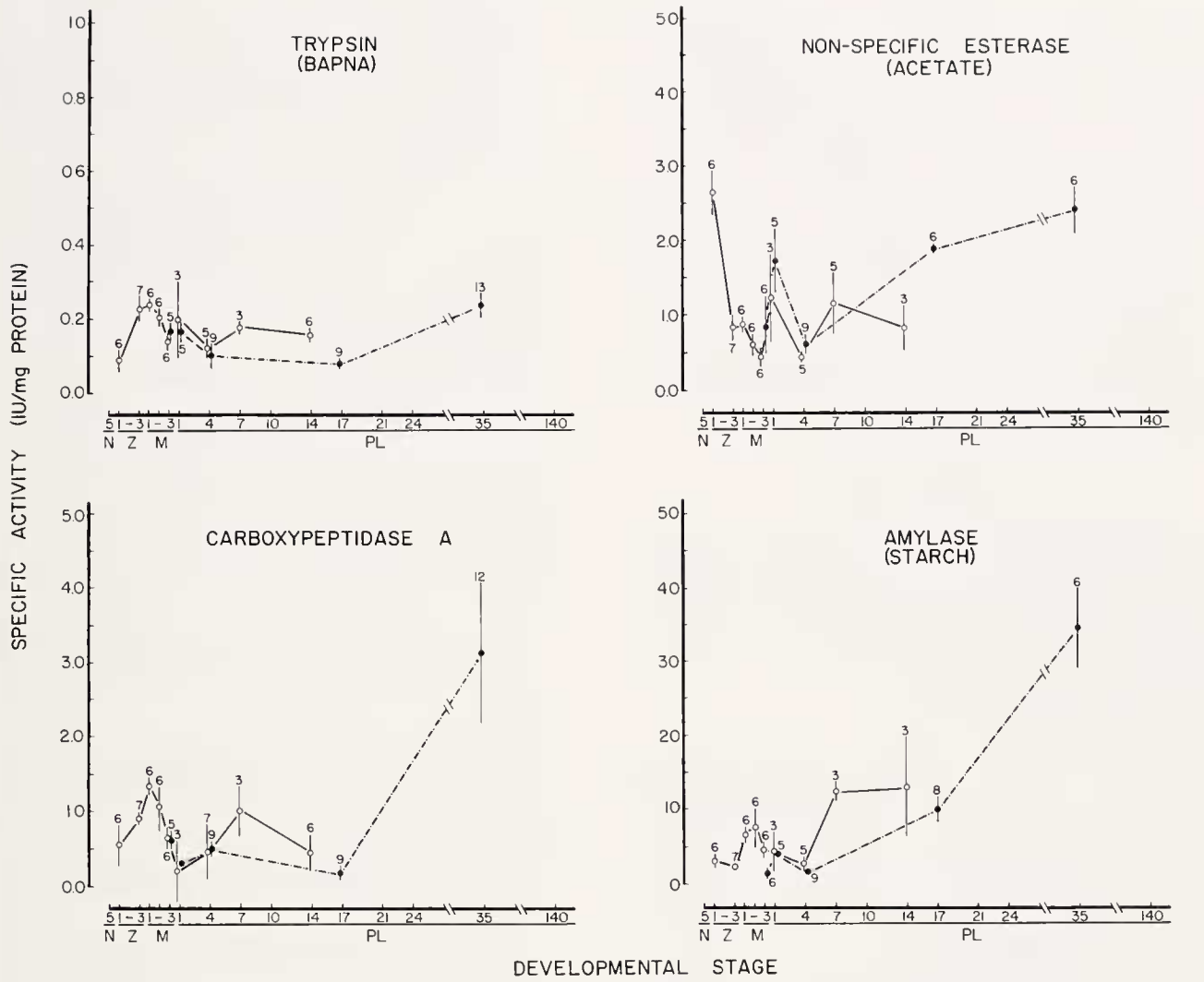

Figure 8. Specific activity of trypsin (BAPNA used as substrate), carboxypeptidase A, non-specific esterase (β-naphthyl acetate used as substrate) and amylase (starch used as substrate) corrected to International Units of activity per mg protein in hepatopancreas, for developmental stages of *Penaeus setiferus*. Concentration of soluble protein was determined for total body; correction to concentration of soluble protein in hepatopancreas was estimated from ratio of hepatopancreas volume to total body volume. Solid *versus* broken lines indicate separate spawnings. Error bars indicate 95% confidence interval about mean activity for each developmental stage. Sample size for each mean is indicated by numbers above or below bars. N, nauplius stage 5; Z, protozoeal stages 1–3; M, mysis stages 1–3; PL, age of postlarvae in days.

LPNA was not membrane-associated (as is aminopeptidase activity in other systems), and (3) substantial activity measured with LPNA came from non-gut tissue. Because there is considerable overlap in the substrates that each esterolytic enzyme can hydrolyze (Nachlas and Seligman, 1949), and because activity of homogenate in the present study decreased as chain length of fatty acid in the substrate increased, activity assayed with each of the three β-naphthol substrates appears to represent a single type of non-specific esterase; lipase activity appears to be absent.

Enzyme activity detected in tissue homogenates may not necessarily represent activity of enzymes that will be secreted into the digestive lumen. In crustaceans, activity for proteases, amylase, chitinase, chitobiase, and non-specific esterase has been found in tissues outside of the gut (Osuna *et al.*, 1977; Trellu and Ceccaldi, 1977; Mykles and Skinner, 1986; Mattson and Mykles, 1987; O'Brien and Skinner, 1987, 1988). Even though such enzymes would contribute to activity assayed in whole-animal homogenates, most enzyme activity detected in *Penaeus setiferus* was restricted to gut tissues. Some diges-

tive enzymes, particularly those associated with lysosomes, are involved in intracellular proeesses only (deDuve and Wattiaux, 1966). Furthermore, because there is no evidence to suggest that digestive enzymes in crustaceans are produeed in a zymogen form (Gates and Travis, 1969; Zwilling *et al.*, 1969; Brockerhoff *et al.*, 1970; Eisen *et al.*, 1973; Zwilling and Neurath, 1981; Vonk and Western, 1984), enzymes that have been synthesized, but have not yet been secreted, also contribute to the enzyme activity measured in tissue homogenates. Nonetheless, intraeellular concentration of digestive enzymes in *Palaemon* directly reflected luminal concentration of enzymes (Rodriguez *et al.*, 1976). Thus, activities assayed in the present study are probable indieators of relative enzyme activities in the lumen.

Acknowledgments

Thanks are extended to A. L. Lawrence and his staff, Texas A&M Shrimp Mariculture Project, for generous provision of *Penaeus setiferus* nauplii and algal cultures. S. C. Hand, University of Colorado, and E. J. DeVillez, Miami University of Ohio, provided valuable adviee on enzyme assays. J. H. Spring, R. C. Brown, J. F. Jackson, B. E. Felgenhauer, K. R. Roberts, and J. L. Staton, University of Southwestern Louisiana, assisted our work in varied capacities. Primary funding was provided under research grant NA85AA-D-SG141 to D. L. Felder and D. L. Lovett from the Louisiana Sea Grant College Program. Additional funds were provided to D. L. Felder by research grants from the Louisiana Education Quality Support Fund, 86-USL(1)-126-07 and 86-LUM(1)-083-13, and from the Coypu Foundation. Louisiana Universities Marine Consortium (LUMCON) Marine Center and the USL Electron Microscopy Center provided valuable technical assistance to the project. This is contribution number 21 from the USL Center for Crustacean Research.

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Appendix 1

Digestive enzymes for which presence or absence (+/−) of activity has been reported in Penaeus *spp. (Activity of general protease is not included)*

Enzyme	Species	Substrate*	Activity	Reference
Trypsin				
	P. setiferus	BAEE	+	Gates and Travis, 1969
		BAPNA	+	Lee, 1984; Lee and Lawrence, 1982, 1985; Lovett and Felder, present paper
		TAME	+	Lovett and Felder, present paper
	P. aztecus	BAPNA	+	Lee, 1984; Lee and Lawrence, 1982
	P. japonicus	BAPNA	+	Cuzon *et al.*, 1980; Galgani, 1983; Galgani *et al.*, 1984, 1985; Tsai *et al.*, 1986; Laubier-Bonichon *et al.*, 1977; Trellu, 1978
		TAME	+	Galgani, 1983; Galgani and Benyamin, 1985; Galgani *et al.*, 1984, 1985; Trellu, 1978
	P. kerathurus	BAPNA	+	Ceccaldi *et al.*, 1983; Galgani, 1983; Galgani *et al.*, 1984
		TAME	+	Galgani, 1983; Galgani and Benyamin, 1985; Galgani *et al.*, 1984; Van Wormhoudt *et al.*, 1972
	P. monodon	BAPNA	+	Galgani, 1983; Tsai *et al.*, 1986
		TAME	+	Galgani, 1983
	P. merguiensis	BAPNA	+	Galgani, 1983
		TAME	+	Galgani, 1983; Galgani and Benyamin, 1985
	P. occidentalis	BAPNA	+	Lee and Lawrence, 1982
	P. penicillatus	BAPNA	+	Tsai *et al.*, 1986
	P. stylirostris	BAPNA	+	Galgani, 1983; Lee and Lawrence, 1982
		TAME	+	Galgani, 1983; Galgani and Benyamin, 1985
	P. vannamei	BAPNA	+	Galgani, 1983; Lee, 1984; Lee and Lawrence, 1982; Lee *et al.*, 1984
		TAME	+	Galgani, 1983; Galgani and Benyamin, 1985
Carboxypeptidase A				
	P. setiferus	BZGPA	+	Gates and Travis, 1973; Lee, 1984; Lee and Lawrence, 1982, 1985; Lovett and Felder, present paper
	P. aztecus	BZGPA	+	Lee, 1984; Lee and Lawrence, 1982
	P. japonicus	FALPP	+	Tsai *et al.*, 1986
		HPLA	+	Galgani, 1983; Galgani *et al.*, 1984
	P. kerathurus	HPLA	+	Ceccaldi *et al.*, 1983; Galgani, 1983; Galgani *et al.*, 1984
	P. merguiensis	HPLA	+	Galgani, 1983
	P. monodon	FALPP	+	Tsai *et al.*, 1986
		HPLA	+	Galgani, 1983
	P. occidentalis	BZGPA	+	Lee and Lawrence, 1982
	P. penicillatus	FALPP	+	Tsai *et al.*, 1986
	P. stylirostris	BZGPA	+	Lee and Lawrence, 1982
		HPLA	+	Galgani, 1983
	P. vannamei	BZGPA	+	Lee, 1984; Lee and Lawrence, 1982
		HPLA	+	Galgani, 1983
Carboxypeptidase B				
	P. setiferus	BZGA	+	Gates and Travis, 1973; Lee, 1984; Lee and Lawrence, 1982, 1985; Lovett and Felder, present paper
	P. aztecus	BZGA	+	Lee, 1984; Lee and Lawrence, 1982
	P. japonicus	BZGA	+	Galgani, 1983; Galgani *et al.*, 1984; Tsai *et al.*, 1986
	P. kerathurus	BZGA	+	Ceccaldi *et al.*, 1983; Galgani, 1983; Galgani *et al.*, 1984
	P. merguiensis	BZGA	+	Galgani, 1983
	P. monodon	BZGA	+	Galgani, 1983; Tsai *et al.*, 1986
	P. occidentalis	BZGA	+	Lee and Lawrence, 1982
	P. penicillatus	BZGA	+	Tsai *et al.*, 1986

Appendix 1 (*Continued*)

Enzyme	Species	Substrate*	Activity	Reference
Carboxypeptidase B (*Continued*)				
	P. stylirostris	BZGA	+	Galgani, 1983; Lee and Lawrence, 1982
	P. vannamei	BZGA	+	Galgani, 1983; Lee, 1984; Lee and Lawrence, 1982; Lee *et al.*, 1984
Arylamidase (Aminopeptidase)				
	P. setiferus	LA	+	Lee, 1984
			−	Lovett and Felder, present paper
		LNA	−	Lovett and Felder, present paper
		LPNA	+	Lovett and Felder, present paper
	P. japonicus	LPNA	+	Galgani, 1983; Galgani *et al.*, 1984; Trellu, 1978
	P. kerathurus	LPNA	+	Ceccaldi *et al.*, 1983; Galgani, 1983; Galgani *et al.*, 1984
	P. merguiensis	LPNA	+	Galgani, 1983
	P. monodon	LPNA	+	Galgani, 1983
	P. stylirostris	LPNA	+	Galgani, 1983
	P. vannamei	LPNA	+	Galgani, 1983
Collagenase				
	P. kerathurus	collagen	+	Ceccaldi *et al.*, 1983; Galgani, 1983; Galgani *et al.*, 1984
Elastase				
	P. kerathurus	elastin	−	Galgani, 1983; Galgani *et al.*, 1984
Pepsin				
	P. setiferus	APAIT	−	Lee, 1984; Lee and Lawrence, 1985; Lovett and Felder, present paper
	P. aztecus	APAIT	−	Lee, 1984
	P. japonicus	APAIT	−	Maugle *et al.*, 1982a
	P. kerathurus	APAIT	−	Galgani, 1983
	P. vannamei	APAIT	−	Lee, 1984; Lee *et al.*, 1984
Low molecular weight protease				
	P. setiferus	?	+	Gates, 1972
		hemoglobin	−	Lee, 1984
	P. kerathurus	gelatin	+	Ceccaldi *et al.*, 1983; Galgani, 1983; Galgani *et al.*, 1984
"Chymotrypsin"				
	P. setiferus	BTEE	+	Lovett and Felder, present paper
		GPANA	−	Lee, 1984; Lee and Lawrence, 1982, 1985; Lee *et al.*, 1984; Lovett and Felder, present paper
	P. aztecus	GPANA	−	Lee, 1984; Lee and Lawrence, 1982
	P. japonicus	BPANA	−	Trellu, 1978
		BTEE	+	Galgani, 1983; Galgani *et al.*, 1984
		SAAPPNA	+	Tsai *et al.*, 1986
		SPNA	−	Galgani, 1983; Galgani *et al.*, 1984
	P. kerathurus	BTEE	+	Galgani, 1983; Galgani *et al.*, 1984; Van Wormhoudt *et al.*, 1972
		SPNA	+	Galgani, 1983; Galgani *et al.*, 1984
	P. merguiensis	BTEE, SPNA	+	Galgani, 1983
	P. monodon	BTEE	+	Galgani, 1983; Tsai *et al.*, 1986
		SAAPPNA	+	Tsai *et al.*, 1986
		SPNA	+	Galgani, 1983
	P. occidentalis	GPANA	−	Lee and Lawrence, 1982
	P. penicillatus	SAAPPNA	+	Tsai *et al.*, 1986
	P. stylirostris	BTEE, SPNA	+	Galgani, 1983
		GPANA	−	Lee and Lawrence, 1982; Lee *et al.*, 1984
	P. vannamei	BTEE	+	Galgani, 1983
		GPANA	−	Lee and Lawrence, 1982; Lee *et al.*, 1984
		SPNA	−	Galgani, 1983

Appendix 1 (*Continued*)

Enzyme	Species	Substrate*	Activity	Reference
Non-specific esterase				
	P. setiferus	α-naphthol	+	Lee, 1984; Lee and Lawrence, 1982
		β-naphthol	+	Lovett and Felder, present paper
	P. aztecus	α-naphthol	+	Lee, 1984; Lee and Lawrence, 1982
	P. occidentalis	α-naphthol	+	Lee and Lawrence, 1982
	P. stylirostris	α-naphthol	+	Lee and Lawrence, 1982
	P. vannamei	α-naphthol	+	Lee, 1984; Lee and Lawrence, 1982
Lipase				
	P. setiferus	olive oil	−	Lovett and Felder, present paper
		tributyrin	+	Lee, 1984; Lee and Lawrence, 1982
	P. aztecus	tributyrin	+	Lee, 1984; Lee and Lawrence, 1982
	P. occidentalis	tributyrin	+	Lee and Lawrence, 1982
	P. stylirostris	tributyrin	+	Lee and Lawrence, 1982
	P. vannamei	tributyrin	+	Lee, 1984; Lee and Lawrence, 1982
Amylase				
	P. setiferus	glycogen	+	Lovett and Felder, present paper
		starch	+	Lee, 1984; Lee and Lawrence, 1982, 1985; Lovett and Felder, present paper
	P. aztecus	starch	+	Lee, 1984; Lee and Lawrence, 1982
	P. indicus	starch	+	Karunakaran and Dhage, 1977
	P. japonicus	glycogen	+	Maugle *et al.*, 1982b, 1983
		starch	+	Cuzon *et al.*, 1980; Laubier-Bonichon *et al.*, 1977; Maugle *et al.*, 1982a, b
	P. kerathurus	starch	+	Van Wormhoudt *et al.*, 1972
	P. occidentalis	starch	+	Lee and Lawrence, 1982
	P. stylirostris	starch	+	Lee and Lawrence, 1982
	P. vannamei	starch	+	Lee, 1984; Lee and Lawrence, 1982
Chitinase				
	P. setiferus	chitin	+	Lee, 1984; Lee and Lawrence, 1985
	P. aztecus	chitin	+	Lee, 1984
	P. vannamei	chitin	+	Lee, 1984
Cellulase				
	P. japonicus	CMC	+	Yokoe and Yasumasu, 1964
Maltase				
	P. japonicus	maltose	+	Maugle *et al.*, 1982b
Sucrase				
	P. japonicus	sucrose	+	Maugle *et al.*, 1982b

* —α-naphthol, α-naphthol acetate; β-naphthol, β-naphthol acetate, laurate, or stearate; APAIT, N-acetyl-L-phenylalanine-L-3,5-di-iodotyrosine; BAEE, N-α-benzoyl arginine ethyl ester; BAPNA, N-α-benzoyl-DL-arginine *p*-nitroanilide·HCl; BPANA, N-benzoyl-DL-phenylalanine-2-naphthylamide; BTEE, N-benzoyl-L-tyrosine ethyl ester; BZGA, hippuryl-L-arginine; BZGPA, hippuryl-L-phenylalanine; CMC, sodium carboxylmethyl cellulose; FALPP, N-(2-furylacryloyl)-L-phenylalanine-phenylalanine; GPANA, gluaryl-L-phenylalanine*p*-nitroanilide; HPLA, hippuryl-L-phenyllactate; LA, L-leucinamide hydrochloride; LNA, L-leucyl-β-naphthylamide hydrochloride; LPNA, L-leucine-*p*-nitroanilide; SAAPPNA, succinyl-L-alanine-L-alanine-L-phenylalanine-L-phenylalanine; SPNA, succinyl-L-phenylalanine nitroanilide; TAME, α-*p*-toluenesulphonyl-L-arginine methyl ester hydrochloride.